Sudan University of Science and Technology
College of Graduate Studies

Molecular Detection of Human Papilloma Virus Type 16 and 18 DNA in Breast Cancer Biopsies from Sudanese Women Attending Omdurman Military Hospital

الكشف الجزيئي عن الحمض النووي لفيروس الورم الحليمي البشري نوع 16 و 18 في خزعات من مريضات سرطان الثدي المترددات على مستشفى أمدرمان العسكري

A dissertation submitted in partial fulfillment of the requirements of M.Sc. degree in Medical Laboratory Science (Microbiology)

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الآية

قال تعالى:

{وَقُلِ اعْمَلُوا فَسَيَرَى اللَّهُ عَمَلَكُمْ وَرَسُولُهُ وَالْمُؤْمِنُونَ ۖ وَسَتُرَدُّونَ إِلَىٰ عَالِمِ الْغَيْبِ وَالشَّهَادَةِ فَيُنَبِّئُكُم بِمَا كُنتُمْ تَعْمَلُونَ}

صدق الله العظيم

[التوبة : 105]

DEDICATION

This humble work is dedicated to . . .

A strong and gentle soul who taught me to trust in Allah ,
believe in hard work and that so much could be done , Mom

For earning an honest living for us and for supporting and
encouraging me to believe in myself , Dad

To my sisters
To my brother

Acknowledgment

All praise is due to **ALMIGHTY ALLAH** to bless my humble efforts with his grace and acceptance. I would like to convey my gratitude to **Dr. Ahmed Ibrahim Hashim** for his supervision and guidance.

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Abstract

Infection of Human Papilloma virus (HPV) has been implicated in the aetiology of a variety of cancer. Several studies have reported the presence of high risk Human Papilloma Virus strain HPV16 and HPV18 in breast cancer biopsies. The aim of this study was to detect Human Papilloma Virus type 16 and 18 DNA in breast cancer biopsies from Sudanese women who attended Omdurman Military Hospital by using multiplex PCR.

This was a retrospective descriptive cross - sectional study during 2015-2016 which was conducted at the research laboratory of the College of Medical Laboratory Science, Sudan University of Science & Technology after getting Ethical clearance from the research committee of College of Post Graduate studies of Sudan University for Science and Technology. A total of 50 breast cancer biopsies (paraffin embedded and previously diagnosed as breast cancer by histological examination) were collected from Omdurman Military Hospital .

Patients demographic data were obtained from hospital records. The age of the patients ranged between 26-85 years with mean age of 51.6 years. The specimens were treated with xylene and different concentrations of ethanol for the removal of paraffin wax. Then the DNA was extracted by salting out method (6 M NaCl), and Multiplex PCR was done for the detection of *E6* gene of HPV 16, and *E7* gene of HPV18.

In this study only 2 (4%) out of the 50 showed positive result for HPV18 , while HPV16 was negative in all of the samples. According to types of breast cancer, HPV18 was found in an invasive ductal carcinoma . The study concluded that there was no significant association between HPV16 , HPV18 and breast cancer. Further studies are required to confirm these results.

المستخلص

عدوى فيروس الورم الحليمي البشري مرتبط في تسبب الأنواع المختلفة للسرطان . وقد كشفت العديد من الدراسات وجود فيروس الورم الحليمي البشري ذات الخطورة العالية نوع 16 و نوع 18 في خزعات سرطان الثدي. الهدف من هذه الدراسة الكشف عن الحمض النووي لفيروس الورم الحليمي البشري نوع 16 و18 في خزعات من مريضات سرطان الثدي المترددات على مستشفى أمدرمان العسكري عن طريق تفاعل البلمره المتسلسل المتعدد.

كانت هذه الدراسة وصفية مقطعية بأثر رجعي خلال الفترة من 2015 الى 2016 و التي أجريت في مختبر الأبحاث بكلية علوم المختبرات الطبية ، جامعة السودان للعلوم و التكنلوجيا بعد أن تم أخذ الموافقة الأخلاقية من لجنة البحوث التي تتبع لكلية الدراسات العليا من جامعة السودان للعلوم و التكنلوجيا . تم جمع 50 خزعة من سرطان الثدي (المغمورة بالبرافين والتي تم تشخيصها سابقا على مصابة بسرطان الثدي عن طريق الفحص النسيجي) من مستشفى أمدرمان العسكري خلال 2015-2016.

تم جمع البيانات الديموغرافية للمرضى من ملفات في سجلات المستشفى. تراوحت أعمار المرضى بين 26-85 سنة مع متوسط العمر 51.6 سنة .تم علاج العينات بالزايلين وتركيزات مختلفه من الايثانول لإزالة شمع البارافين . ثم تم استخراج الحمض النووي باستخدام الطريقة المالحة . و تم اجراء تفاعل البلمرة المتسسل المتعدد للكشف عن جين *E6* لفيروس الورم الحليمي البشري نوع 16 و جين *E7* لفيروس الورم الحليمي البشري نوع 18.

في هذه الدراسة فقط أعطت 2 عينة (4٪) من أصل 50 أظهرت نتيجة إيجابية لفيروس الورم الحليمي البشري 18، في حين أن كل العينات سلبية لفيروس الورم الحليمي البشري 16 ، بالرغم من أن نوع فيروس الورم الحليمي البشري 16 لم يتم اكتشافه . بناءا" على انواع سرطان الثدي ، نوع فيروس الورم الحليمي البشري 18 تم اكتشافه في سرطان الأقنية المجتاحة . خلصت الدراسة الى انه لا يوجد علاقة بين فيروس الورم الحليمي البشري نوع 16 و 18 و سرطان الثدي . المزيد من الدراسات مطلوبة لتأكيد هذه النتائج.

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1.1 Introduction

The human papilloma virus (HPV) is one of the most common causes of sexually transmitted disease in both men and women around the world, especially in developing countries, where the prevalence of asymptomatic infection varies from 2 to 44%, depending on the population and studied region (De Sanjosé *et al*., 2007).

Most HPV infection is transient and some studies show that the majority of sexually active individuals are exposed to and acquire infection from this virus at some phase in their lives (Baseman and Koutsky, 2005 ; Trottier and Franco, 2006). HPV infection is more prevalent in young adults, at the beginning of their sexual activity, with a subsequent decline in the prevalence rate with increasing age, likely as a result of development of an immune response against the virus and reduction of sexual activity (Philip *et al*., 2005 ; Fernandes *et al*., 2009 ; Chan *et al*., 2010).

Today, more than 150 different HPV types have been recorded and about 40 can infect the epithelial lining of the anogenital tract and other mucosal areas of the human body. Based on their association with cervical cancer and precursor lesions, HPVs can also be classified as high-risk (HR-HPV) and low-risk (LR-HPV) oncogenic types. LR-HPV types, such as HPV 6 and 11, can cause common genital warts or benign hyperproliferative lesions with very limited tendency to malignant progression, while infection with HR-HPV types, highlighting HPV 16 and 18, is associated with the occurrence of pre-malignant and malignant cervical lesions (Bosch *et al*., 2002 ; Muñoz *et al.*, 2003 ; Bosch *et al*., 2008).

High risk human papillomavirus (HR-HPVs) are carcinogenic viruses which are primarily associated with cervical cancer but are also linked with other anogenital cancers and cancers of other organ sites, such as oral cavity, esophagus, nasopharyngeal and laryngeal carcinoma and possibly in retinoblastoma (Hedau *et al*., 2011). Correlation of DNA identification of HPV and breast cancer ranges in variability from 0–86% of cases but the mechanism by which the virus reaches the breast has not been clearly identified (De León *et al*., 2009). Various subtypes of HPV including HPV-11, HPV-16, HPV-18, HPV-33, HPV-58, HPV-59, HPV-73, and HPV-82 are candidate subtypes of HPV associated with breast cancers. On the contrary, some studies did not detect HPV infection in breast cancers (Lindel *et al*., 2007 ; Vernet-Tomas *et al*., 2015).

According to studies carried out in China, Australia, Italy, Japan, the USA, Norway, Greece, Korea, Mexico and Taiwan, HPV infection was found among women with breast cancer (Lawson and Heng, 2010). In a systematic review conducted in Europe, North America and Australia, a correlation was found between HPV and breast cancer (Simões *et al*., 2010).

1.2 Rationale

Despite much research, the role of human papilloma virus in malignant transformation of mammary gland cells is still unclear and the available research results are inconclusive. There are many publications in which the presence of virus was excluded in women with breast cancer (Mou *et al*., 2011; Hedau *et al*., 2011), as well as those where the relationship has been demonstrated (Lawson *et al*., 2009 ; Simoes *et al*., 2012). The large variability of results may be due to different tests used to detect the presence of the virus, the selection of appropriate primers, as well as the biological material taken to isolate the genetic material of the virus. The mechanism for inducing malignant transformation in cells has also been discussed. While the role of oncogenic proteins *E6* and *E7* is evidenced in malignant transformation of epithelial cells of the cervix, some researchers have cast doubt on this mechanism in the case of breast cancer (Frega *et al*., 2012).

The suspicion that HPVs may also have a role in human breast cancer is based on the identification of HPVs in human breast tumours and the immortalisation of normal human breast cells by HPV 16 and 18 (Band *et al.*, 1990 ; De Villiers *et al.*, 2005). Human papilloma virus 11, 16 and 18 have been identified in breast cancer in US and Brazil separtely (Liu *et al.*, 2001; Damin *et al.*, 2004).The aim of this study was to detect HPV in breast cancer patients in Khartoum State.

1.3 Objectives

1.3.1 General objectives

To detect Human Papilloma Virus type 16 and 18 DNA using molecular techniques in breast cancer tissue biopsies from Sudanese Women attending Omdurman military hospital 2015- 2016.

1.3.2 Specific objectives

1- To extract HPV type 16 and 18 DNA from breast cancer biopsies of Sudanese Women using Salting out method.
2- To detect HPV type 16 and 18 using multiplex PCR technique.
3- To correlate between the breast cancer , demographic data , type of breast cancer, breast cancer and HPV type 16 & 18.

CHAPTER TWO

LITERATURE REVIEW

CHAPTER TWO

2.LITERATURE REVIEW

2.1 Biological properties of HPV

Human papilloma viruses belongs to the papilloma virus family, *Papillomaviridae*, capable of infecting humans and the most prevalent sexually transmitted viral infection. It is estimated that 80% of sexually active adults have been infected with at least one HPV type (Baseman and Koutsky, 2005).

2.1.1 Structure of viral particle and regulation of gene expression

The human papilloma virus (HPV) is a relatively small non-enveloped virus that contains a double-stranded closed circular DNA genome, associated with histone-like proteins and protected by a capsid formed by two late proteins, *L1* and *L2*. Each capsid is composed of 72 capsomeres, each of which is composed of five monomeric of 55kDa units that join to form a pentamer corresponding to the major protein capsid, *L1*. The *L1* pentamers are distributed forming a network of intra- and interpentameric disulfide interactions which serve to stabilize the capsid (Sapp *et al*., 1995). In addition to *L1*, minor capsid proteins with approximately 75kDa exist within the virion and are called the *L2* protein. To assemble the viral capsid, the pentamers join to copies of *L2* that occludes the center of each pentavalent capsomere (Jo and Kim, 2005; Buck *et al*., 2008 ; Conway and Meyers, 2009). Thus, each virion contains 72 copies of the *L1*, the major component of the capsid, and a variable number of copies of *L2* , a secondary component of the viral capsid, forming a particle with icosahedra symmetry and approximately 50 to 60 nm in diameter (Burd, 2003 ; Longworth and Laimins, 2004 ; zur Hausen, 2009). The viral genome of the HPV consists of a single molecule of double-stranded and circular DNA, containing approximately 8000 base pairs and harboring an average of 8 open reading frames (ORFs) (Jo and Kim , 2005 ; Zheng and Baker , 2006).

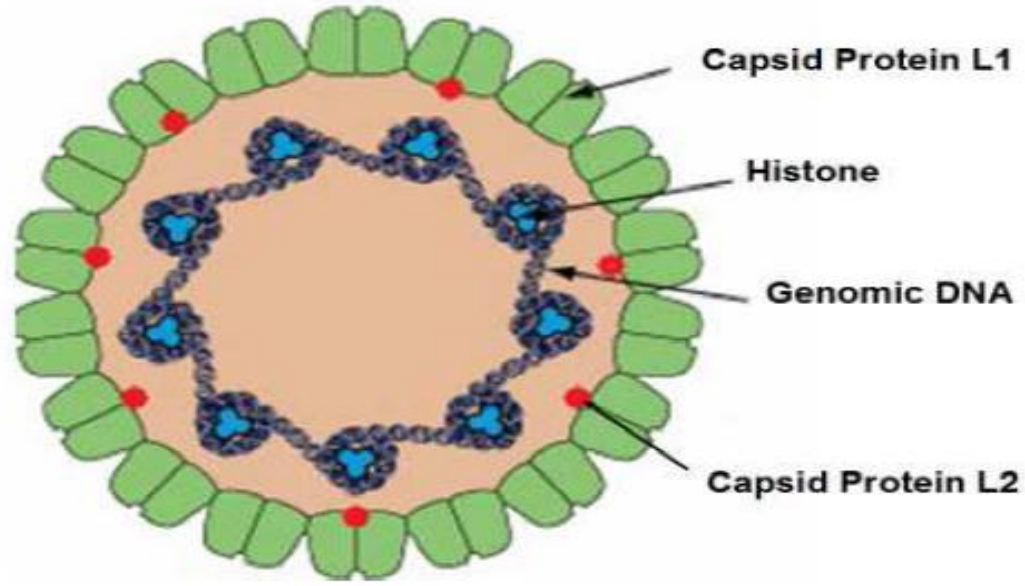
In a functional point of view, the HPV genome is divided into three regions. The first is a noncoding upstream regulatory region (URR) or long control region (LCR) that has regulatory function of the transcription of the *E6* and *E7* viral genes; The second is an early region (*E*) , consisting of six ORFs: *E1*, *E2*, *E4*, *E5*, *E6*, and *E7*, which encodes no structural proteins involved in viral replication and oncogenesis. The third is a late (*L*) region that encodes the *L1* and *L2* structural proteins. The LCR region of the anogenital HPVs ranges in size between 800-900 pb, representing about 10% of the genome, and varies substantially in nucleotide composition between individual HPV types (Fehrmann and Laimins , 2003 ; Jo and Kim, 2005).

Only one strand of the double-stranded DNA serves as the template for viral gene expression, coding for a number of polycistronic mRNA transcripts (Stanley *et al*., 2007). The regulation of viral gene expression is complex and controlled by cellular and viral transcription factors. Most of these regulations occur within the LCR region, which contains cis-active element transcription regulators. These sequences are bound by a number of cellular factors as well as the viral *E2* product (zur Hausen, 1996). A large number of cellular transcription factors have been identified and the dysfunction of some of them appears to play a significant role in papillomavirus-linked carcinogenesis (Thierry *et al*., 1992 ; Hamid *et al*.,2009).

The transcription start sites of viral promoters differ depending on the virus type, but, in all types, promoter usage is keratinocyte differentiation-dependent (Smith *et al*., 2007). The replication origin and many transcriptional regulatory elements are found in the upstream LCR region. The virus early promoter, differentiation-dependent late promoter, and two polyadenylation signals define three general groups of viral genes that are coordinately regulated during host cell differentiation The *E6* and *E7* genes maintain replication competence. *E1*, *E2*, *E4*, *E5*, and *E8* are involved in virus DNA replication, transcriptional control, beyond other late

functions and *L1* and *L2*, responsible for the assembly of viral particles (Bodily and Laimins, 2011).

The regulation of expression of the late genes in genital HPVs is not well understood. However, it has been shown that the second, or later, promoter is initiated in a differentiation-dependent manner, and thus is activated only when cells are grown in the host's stratifying/differentiating tissue. Once activated, the later promoter directs transcription from a heterogeneous set of start sites and will serve to produce a set of transcripts that facilitate the translation of *L1* and *L2* proteins (Smith *et al*., 2007; Conway and Meyers, 2009). Activation of the later promoter is accompanied by acceleration of viral DNA replication and by high levels of viral protein expression. As a result, virus copy-number amplifies from 50 copies to several thousands of copies per cell. So when a late promoter is activated, the expression of genes will occur, encoding the structural proteins *L1* and *L2*, which join to assemble the capsids and to form virions (Stanley *et al*., 2007).



The structure of HPV (Fernandes and Fernandes, 2012)

2.1.2 Functions of viral proteins

2.1.2.1 *E1* Protein

The *E1* protein represents one of the the most conserved proteins among different HPV types. It has DNA-binding functions and a binding site in the origin of replication localized in the long control region (LCR) region. It assembles into a hexameric complex, supported by the *E2* protein, and the resultant complex has helicase activity and initiates DNA bidirectional unwinding, constituting a prerequisite for viral DNA replication (Wilson *et al*., 2005).

2.1.2.2 *E2* Protein

The *E2* open reading frame of HPV gives rise to multiple gene products by alternative RNA splicing. The proteins derived from the *E2* gene are involved in the control of viral transcription, DNA replication, and segregation of viral genomes (McPhillips *et al*., 2006 ; Kadaja *et al*., 2009).

2.1.2.3 *E4* Protein

The *E4* protein has been suggested that *E4* may have an important role in favoring and supporting the HPV genome amplification, besides regulating the expression of late genes, controlling the virus maturation, and facilitating the release of virions (Longworth and Laimins, 2004).

2.1.2.4 *E5* Protein

HPV *E5* can enhance the transforming activity of *E6* and *E7*, suggesting that it may have a supportive role in tumor progression, also been reported to alter the activity of the epidermal growth factor receptor (EGFR), in addition to reducing the surface levels of major histocompatibility complex (MHC) class I proteins, modulating the MAPK pathway and altering the levels of caveolin 1 (Moody and Laimins, 2010).

2.1.2.5 *E6* Oncogene

The best known property of the *E6* proteins of HR-HPVs is the ability to bind and degrade the tumorsuppressor protein p53, through the recruitment of the *E6*-associated protein (*E6*-AP), a cellular *E3* ligase that does not bind to p53 in the absence of *E6*. Both *E6* proteins from HR -HPV and LR-HPV bind to p53, but the interaction is stronger in HR-HPV (Lechner *et al*., 1994).

The *E6* protein can overcome the cell arrest and proapoptotic activities of p53 by targeting p53 for degradation, inactivating the Mdm2 pathway. *E6* can also inhibit the transcriptional activities of p53 independently of *E6*-AP (Thomas *et al*., 2005).

The degradation or blocking of the p53 function inhibit apoptotic signaling that would eliminate the HPV infection cell. There are two major apoptotic pathways that can be triggered by different stresses: the extrinsic and intrinsic pathways. The *E6* protein is able to disrupt both pathways to facilitate a cytoprotective environment and prevent cell death (Howie *et al*., 2009).

High-risk HPV *E6* proteins induce rapid degradation of p53 through ubiquitin-dependent proteolysis, so HPV infected cells have lower levels of unstable p53 (Scheffner *et. al.*, 1990). This results in bypassing the normal growth arrest signals at the G1/S and G2/M checkpoints leading to accumulation of mutations and later transformation (Fehrmann and Laimins, 2003).

2.1.2.6 *E7* Oncogene

HPV *E7* protein has a short half-life that rapidly degraded by proteasomal degradation (Munger *et al.*, 1989a ; Munger *et al.*, 2001). The high risk *E7* proteins associate with the retinoblastoma (pRb) to facilitate progression into S phase (Vousden, 1994). *E7* subvert cellular targets such as pRB are indispensable for HPV replication and the full viral life cycle (Flores *et al*., 2000). A single *E7* polypeptide inactivates multiple molecules of cellular target proteins HPV *E7* could interfere with the ability of p53 to induce G1 growth arrest (Hickman *et al.*,

1994). HPV-16 *E7* forms complexes with hypophosphorylated pRb, leading to its inactivation and permits S phase entry (Cobrinik *et al.*, 1992).

E7 proteins encoded by high-risk and low-risk HPVs differ in several biochemical and biological properties. Low risk HPV-6 and -11 *E7* proteins bind pRb with a lower efficiency than the high risk HPV-16 and -18 E*7* proteins, but fail in targeting it for degradation and do not transform the cell (Munger *et al.,* 1989b ; Barbosa *et al.*, 1990). Sequence comparison revealed that high risk HPV E*7* have an aspartic acid residue in high-risk HPV *E7* proteins (Asp 21 in HPV-16 *E7*) versus a glycine residue in the low-risk HPV *E7* sequence Gly 22 in HPV-6 *E7*) (Sang and Barbosa, 1992).

2.1.2.7 *L1* Protein

The *L1* protein is highly immunogenic and has conformational epitopes that induce the production of neutralizing type-specific antibodies against the virus, which prevent the infection (Carter *et al*., 2003)

2.1.2.8 *L2* Protein

L2 contributes to the binding of virion in the cell receptor, favoring its uptake, transport to the nucleus, and delivery of viral DNA to replication centers. Besides, *E2* helps the packaging of viral DNA into capsids and, due to the presence of a usual neutralization epitope in *L2* proteins of many papilloma viruses, it may be instrumental in conferring immunity across different types of HPV. *L2* also contributes to the interaction of virion in the cell surface (Schiller *et al*., 2010).

2.2 Life cycle of HPV

The HPV life cycle begins with infection of stem cells in the basal layer of the epithelium. After the entry in the cells, the virus requires the expression of *E1* and *E2* genes to maintain a low number of copies of genome. These proteins bind to the viral origin of replication and recruit cellular DNA polymerases and other proteins necessary for DNA replication (Hamid *et al*., 2009). In the suprabasal

layer, the expression of genes *E1, E2, E5, E6* and *E7* contributes to the maintenance of the viral genome and induces cell proliferation , increasing the number of HPV-infected cells in the epithelium, resulting in a higher number of cells that will eventually produce infectious virions (Hamid *et al*., 2009 ; Lazarczyk *et al*., 2009). In the more differentiated cells of this same layer of the epithelium occurs the activation of differentiation-dependent promoter and maintenance of gene expression *E1, E2, E6* and *E7*. Furthermore, there will be activation of the expression of *E4* gene, whose product will induce amplification of the viral genome replication, greatly increasing the number of virus copies per cell, at the same time that occurs the expression of genes L*1* and L*2* (Nakahara *et al*., 2005 ; Lazarczyk *et al*., 2009). In the granular layer, the products of late genes, the major and minor proteins of the viral capsid, *L1* and *L2* respectively, gather to assembly of the viral capsids and formations of virions, which reach cornified layer of the epithelium and are released (Lazarczyk *et al*., 2009).

For a better understanding, the life cycle of HPV was divided into two parts: a maintenance phase and differentiation-dependent phase (Bodily and Laimins, 2011).

2.2.1 Maintenance phase

HPV virions infect cells in the basal epithelial layer that become exposed through microlesions. The viral capsid binds initially to the basal cell layer and infection occurs when activated keratinocytes move into the wound, to the upper layers of the epithelium (Kines *et al*., 2009). HPV genomes replicate in the nucleus of the basal cell layer, where the viral replication is considered nonproductive and the virus establishes itself as a low-copynumber episome by using the host DNA replication machinery (Moody and Laimins, 2010). In this way, viral proteins are expressed at very low levels in undifferentiated cells, and this contributes to immune avasion and persistence (Bodily and Laimins, 2011).

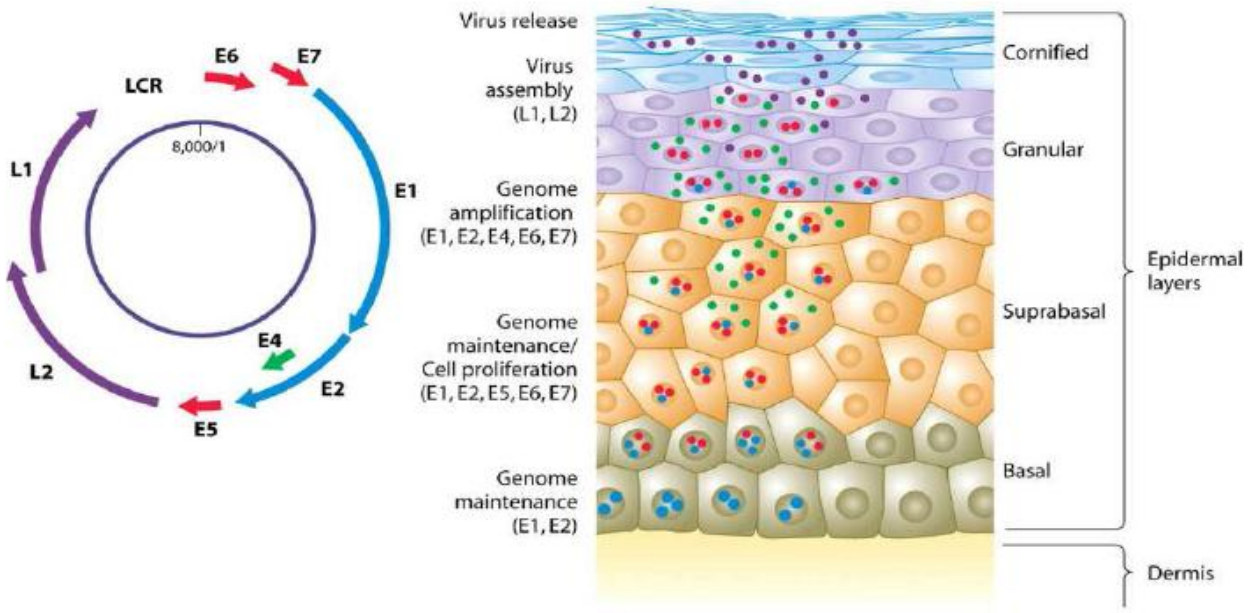
The maintenance of the viral episome in basal cells is the basic function of the early or maintenance phase of the viral cycle. The expression of *E6* , *E7*, *E1*, and *E2* are necessary for continued episomal maintenance. *E1* and *E2* cooperate to initiate viral DNA replication, whereas *E6* and *E7* modulate cell-cycle regulators to maintain long-term replication competence (Conger *et al*., 1999). The *E2* protein is probably a major regulator of this process because it is able to make both positive and negative control of the early viral promoter that regulates expression of *E6*, *E7*, and *E1* as well as *E2* itself (Steger *et al*., 1997). Following this establishment phase, viral DNA is replicated coordinately with host cell chromosomes, and virus genomes are distributed to the daughter cells. However, in the differentiated keratinocytes of the suprabasal layers of the epithelium, the virus switches to a rolling-circle mode of DNA replication, amplifying its DNA to a high copy number, synthesizing capsid proteins, and assembling the viral particle (Flores *et al*., 1999). HPV replication begins when the host cell factors interact with the LCR region of the HPV genome and begin the transcription of the early viral genes, highlighting the *E6* and *E7*. The viral *E6* and *E7* gene products deregulate the cell cycle, subverting the cell growth regulatory pathways and modifying the cellular environment in order to faclitate viral replication in a cell that is terminally differentiated and has exited the cell cycle (Syrjânen and Syrjânen, 1999).

2.2.2 Differentiation-dependent phase

During the maintenance phase in undifferentiated cells, viral proteins are expressed in extremely low levels. However, when HPV-infected cells leave the basal layer, they undergo differentiation and high levels of viral proteins synthesis are induced. This restriction of viral protein synthesis to highly differentiated cells delays the expression of viral antigens to locations less susceptible to the host immune response (Frazer, 2009).

This compartimentalization of gene expression by HPVs constitutes an important strategy to sustain long-term infection, but it creates some problems for the virus. To solve this, the virus forces the cell to remain active in the cell cycle, enabling productive replication in differentiating cells. The viral protein *E7* is responsible for maintaining the replication competence in differentiated cells and this is accomplished in part by inactivation of pRB family members (Münger *et al*., 2004). The activation of the late viral promoter in response to host-cell differentiation occurs in the vicinity of the spinous epithelial layer and is responsible for high levels of viral protein expression. As a result, the virus copy-number amplifies from 50-200 copies to several thousands of copies per cell (Bedell *et al*., 1991).

The viral proteins *E1, E4*, and *E5* contribute to the activation of late viral functions upon differentiation (Fehrmann *et al*., 2003 ; Wilson *et al*., 2005). The *E2*-mediated down-regulation of *E6* and *E7* transcription results in the release of the p53 and pRB cellular proteins, and allows the normal differentiation process of the host cell. Then, a putative late promoter activates the capsid genes, *L1* and *L2*. Finally, the viral particles are assembled in the nucleus, and the complete virions are released when the cornified layers of the epithelium are shed. The virions are shed in an environment with desquamated cells in the absence of lysis or necrosis, and this further contributes to virus persitence because it avoids inflammation (Stanley, 2008). Most women infected with a specific HPV type will not show evidence of that same type after 6-12 months. It is not known whether the HR-HPV can be detected for periods similar to those for LR-HPV. Some studies show similar duration (Richardson *et al*., 2003), but others reveal longer durations of infection for HR-HPV types (Ho *et al*., 1998 ; Franco *et al*., 1999). It appears that HR-HPV, particularly HPV16, has a longer time to clearance and is more likely to develop persistent infection (Richardson *et al*., 2003).



HPV Cycle life (Lazarczyk *et al*., 2009).

2.3 Pathogenesis

The infection with HR-HPV typically lasts from 12 -18 months and is eventually cleared by the immune system (Richardson *et al*., 2003).

However, approximately 10% of women fail to clear HPV infections, resulting in a persistent infection.The main consequence of persistent infection with HR-HPV is the development of lesions that may progress to malignancy, and this constitutes the most important risk factor for the development of cervical cancer (Stanley 2008; Moody and Laimins, 2010 ; Bodily and Laimins, 2011).

Details about the immune response that results in clearance of HPV infection are still unknown. HPV clearance seems to result in long-term humoral and/or cellular protection against re-infection by the same HPV type; whether the protection is lifelong is not known (Stanley, 2006). Although the term clearance is used when an HPV infection can no longer be detected using sensitive test methods, the HPV presence might not be completely discarded because the latent state of HPV is still poorly understood. Reappearance of HPV from latency even in the absence of

definite immunosuppression is common, but most cases are probably benign (Gonzalez *et al.*, 2010).

It is important to remember that it is not easy to characterize a persistent HPV infection and differentiate persistent infection from healing followed by re-infection, although re-infection with the same HPV type appears to be uncommon. Many studies classify HPV infection as persistent if the HPV was detected in two consecutive follow-up visits 4-6 months apart. However, because the interval between follow-up visits varies among studies and there are many unknown questions regarding the natural history of HPV, it is complicated to distinguish persistent and transience infections. Furthermore, an undetectable HPV infection could be a period of viral latency, in which the HPV levels are below the detectable threshold of current HPV DNA assays, instead of representing a cleared host (Baseman and Koutsky, 2005).

The persistent nature of HPV infection and DNA viral integration into the genome of the cell contributes to increasing the risk of high-grade and malignant lesions because of genomic instability generated. *E6* and *E7* can induce centrosomal abnormalities resulting in abnormal centrosome reduplication, leading to abnormal numbers of centrosomes . Furthermore, abrogation of cell-cycle checkpoints through the targeting of p53 and pRB family members allows retention of cells with chromosomal abnormalities (Münger *et al*., 2004). This can result in genetic changes that accumulate over an extended period of time until resulting in a combination of genetic abnormalities, allowing cancer development (Bodily and Laimins, 2011).

In benign and malignant HPV lesions, the cellular proliferation increases the demand for nutrients, generating a competition for nutrients and oxygen. To overcome this constraint, both HR-HPV and LR-HPV *E7* proteins enhance the levels of the transcription factor Hypoxia-inducible factor-1 (HIF-1), as well as

induce the increased expression of HIF-1 target genes under hypoxia conditions (Nakamura *et al*., 2009). The enhancement of HIF-1 activity results in an increased transcription of a subset of genes that favor angiogenesis, and this induction of angiogenesis is crucial to both persistence and growth of HPV lesions (Bodily and Laimins, 2011).

2.4 Breast Cancer

For many years the incidence of breast cancer remained at a high level. There are many risk factors which increase the probability of occurrence of the development of neoplastic transformation (Andrejuk *et al*., 2013). Many epidemiological studies point out that a family history of breast cancer is a reproducible predictor of breast cancer risk (Palmer *et al*., 2009).Observation of the increased risk of breast cancer for women with positive family histories of breast cancer leads to further sub-classifications into hereditary (familial) or sporadic cases. Some risk factors including obesity have been related to the pathogenesis of breast cancer (Cleary and Grossmann, 2009) .

Many other factors, including estrogen levels, estrogen receptors, and the adipokines leptin and adiponectin appear to be important in the mechanism of these increased risk factors. Moreover, it was suggested that exogenous and endogenous modulators of oxidative stress may modify the association between the myeloperoxidase polymorphism and breast cancer risk (He *et al*., 2009).

However, the molecular events in the genesis of this disease are not entirely clear. The reports indicate hereditary breast cancers are responsible for 4–10% of breast carcinomas (Arver *et al*., 2000 ; Rahman and Scott, 2007).

Several studies have reported that some viruses such as Epstein-Barr virus (EBV), Mouse mammary tumor virus (MMTV) and HPV have important roles in developing breast cancer (Lawson and Heng, 2010 ; Ashrafi *et al*., 2005).

2.5 Breast cancer and Human Papillomavirus infection

Breast tumorigenesis might be promoted by viral infection. High-risk HPV such as HPV type 16(HPV16), HPV type 18(HPV18) were associated with cervical cancer, anogenital cancers and cancers of other organs (zur Hausen, 2009; Acevedo *et al*., 2004). Factors that favor a small proportion of HPV16 infections to progress to cancer are still poorly understood, but the genetic variation has implicated a role of HPV16 in previous study (Schiffman *et al*., 2010 ; Gheit *et al*., 2011 ; Di lonardo *et al*., 1992).

There were increasing studies reporting on the involvement of HPV DNA in breast cancer in recent years, but the conclusions remained to be highly controversial. Di Lonardo et al. first reported the detection of HPV16 DNA in 29% of 17 patients with breast cancer by polymerase chain reaction (PCR)

CHAPTER THEREE

MATERIALS AND METHODS

CHAPTER THEREE

3.MATERIALS AND METHODS

3.1 Study design

This study was a retrospective descriptive cross-sectional study.

3.2 Study area

The study was conducted in the Omdurman military hospital in Khartoum State.

3.3 Study population and duration

Fifty paraffin blocks previously diagnosed as breast cancer from women who attended to the Military Hospital during 2015 – 2016.

3.4 Inclusion criteria

Breast biopsies which were previously diagnosed as breast cancer.

3.5 Exclusion criteria

Breast biopsies which were previously diagnosed as benign tumor.

3.6 Ethical clearance

Ethical clearance was obtained from the research committee of College of Post Graduate studies of the Sudan University of Science and Technology.

3.7 Data collection

Patients demographic data was collected from patients files in the hospital records.

3.8 Experimental work

3.8.1 Processing of specimens

From each breast cancer patient's paraffin embedded block , small sections of 10µm were collected into a screw capped Eppendorf tube. To avoid cross

contamination, each block was cut with new gloves and new disposable microtome blade.

3.8.2 De-paraffinization and re-hydration of sections

One ml of xylene was added to a screw capped Eppendorf tubes containing sections of breast cancer embedded in paraffin then were vortexed and left for 10 min. Then sections were centrifuged at 14,000 rpm for 10 min and the supernatant was aspirated, this step was repeated twice. Then one ml of different ethanol concentrations (100%, 80%, 50% Appendix III) were added to each sample respectively and centrifuged at 14,000rpm for 10 min and the supernatant was aspirated. Then one ml of dH_2O was added and were incubated at 4^oC overnight (GMB, 2010).

3.8.3 DNA Extraction

3.8.3.1 Digestion of protein

The pellet obtained from previous steps was treated with 700µl nucleic lysis buffer (Appendix III) and 50µl of proteinase K, and was incubated at 65^oC for 24 hours. An additional 50µl proteinase K was added next day and was incubated for an additional 24 hours at 65^oC (GMB, 2010).

3.8.3.2 Precipitation and isolation of DNA

Ten µl of RNAse was added followed by addition of 250µl of 6 M NaCl (Appendix III) and was let at room temp for 10 min. Then was centrifuged at 14,000 for 10 min, and the supernatant was transferred to a clean tube. Then 1ml of ice-cold (or -20^oC) 100% ethanol was added, the sample was mixed carefully and placed at -20^oC for 20 min. Then was pelleted at 14,000 rpm for 10 min and the supernatant was carefully discarded. The pellet was washed with 1.5 ml of 70% ethanol and centrifuged at 14,000 rpm for 10 min then the supernatant was

carefully discarded and the pellet was air dried on the bench top for 10 to 15 min. Finally 50 µl of TE buffer (Appendix III) was added and was stored at -20^{O}C for further usage (GMB, 2010).

3.8.4 Polymerase chain reaction – PCR

All DNA samples were screened by HPV16 E*6* primer (diagnostic band 315bp) and HPV18 E*7* primer (diagnostic band 152bp) were synthesized by (Macrogen, Korea).

PCR was done by multiplex PCR, amplification was done using TECHNE® Ltd peltier thermal cycler (Germany), DNA amplifies was done using Maxime PCR Premix kit (iNtRON, Korea)

3.8.4.1 PCR Protocol: HPV16 *E6* and HPV18 *E7*

3.8.4.1.1 HPV16 *E6* Primer sequence (Appendix III)

Forward 5'-CTG CAA GCA ACA GTT ACT GCG ACG-3'
Reverse 5'-CAT ACA TCG ACC GGT CCA CC-3' (Li *et al.*, 2015)

3.8.4.1.2 HPV18 *E7* Primer sequence (Appendix III)

Forward 5'-GAG CCG AAC CAC AAC GTC AC-3'
Reverse 5'-GGA TGC ACA CCA CGG ACA CA-3' (Li *et al.*, 2015)

3.8.4.1.3 PCR mix per sample

Maxime PCR PreMix Kit.(Appendix II)

HPV16 *E6* forward primer 0.5µl

HPV16 *E6* reverse primer 0.5µl

HPV18 *E7* forward primer 0.5µl

HPV18 *E7* reverse primer	0.5µl
Sample DNA template	4µl
dH_2O	14µl
Total volume	20µl

3.8.4.1.4 PCR program:

Initial denaturation		94ºC/2min
40 cycles	Denaturation	94ºC/20sec
	Annealing	56ºC/20sec
	Extension	72ºC/25sec
Final extension		72ºC/5min

3.8.4.1.5 Gel electrophoreses of the PCR products:

Six µl of the PCR products were loaded in 2% agarose gel (Appendix III).

Agarose gel 2% was used for HPV16 *E6* (diagnostic band 315bp), and HPV18 *E7* (diagnostic band152bp). 10 microliters of each PCR products was loaded on agarose gel in the tank submerged with loading buffer (Appendix III) and then run at 100 volt for 15 minutes then at 75 volt for 10 minutes. For each gel a DNA ladder marker, positive control and a negative controls were loaded and then the samples. The gel transferred into a gel documentation system, to be viewed under UV light and photographed. A PCR results were regarded as positive if it has the same size as the expected size of the diagnostic band as read by the ladder marker and in comparison to the positive control. The positive controls were used to be compared with PCR product and to insure efficiency of PCR kits.

3.9 Quality control

Standard procedures for preventing contamination were strictly applied. New gloves for each sample and step and clean lab coat was used. DNA samples and PCR products were kept in separate boxes. The PCR kits were divided into three separate sets (aliquots). Ice Block was used during preparation of PCR mix inside sterile lamina flow hood. Distilled water for antibiotics were used each time and were kept closed and frozen until needed .All pipette tips and Eppendorf tubes were autoclaved. No mobiles or chat with colleagues during PCR. The condition of each experiment was written and each experiment was photographed and the results were recorded. The bench and the hood were cleaned by ethanol before and after work and a UV light was set on overnight. Each DNA sample and PCR product were fully labeled using water proof permanent fine tip black marker (sample number, PCR reaction and date).

3.10 Data analysis

Results and data were analyzed using Statistical Package of Social Science (SPSS) version 11.5 by Chi-square test.

CHAPTER FOUR

RESULTS

CHAPTER FOUR

4.RESULTS

Formalin embedded tissues blocks (n = 50) of Sudanese women with histologically confirmed breast cancer (BC) were selected. Their mean and age range were 51.6 years and 26 to 85 years, respectively (Fig. 4.1). 56% (28/50) of the patients was below 51years, and 44 % (22/50) their ages were above 52 years.

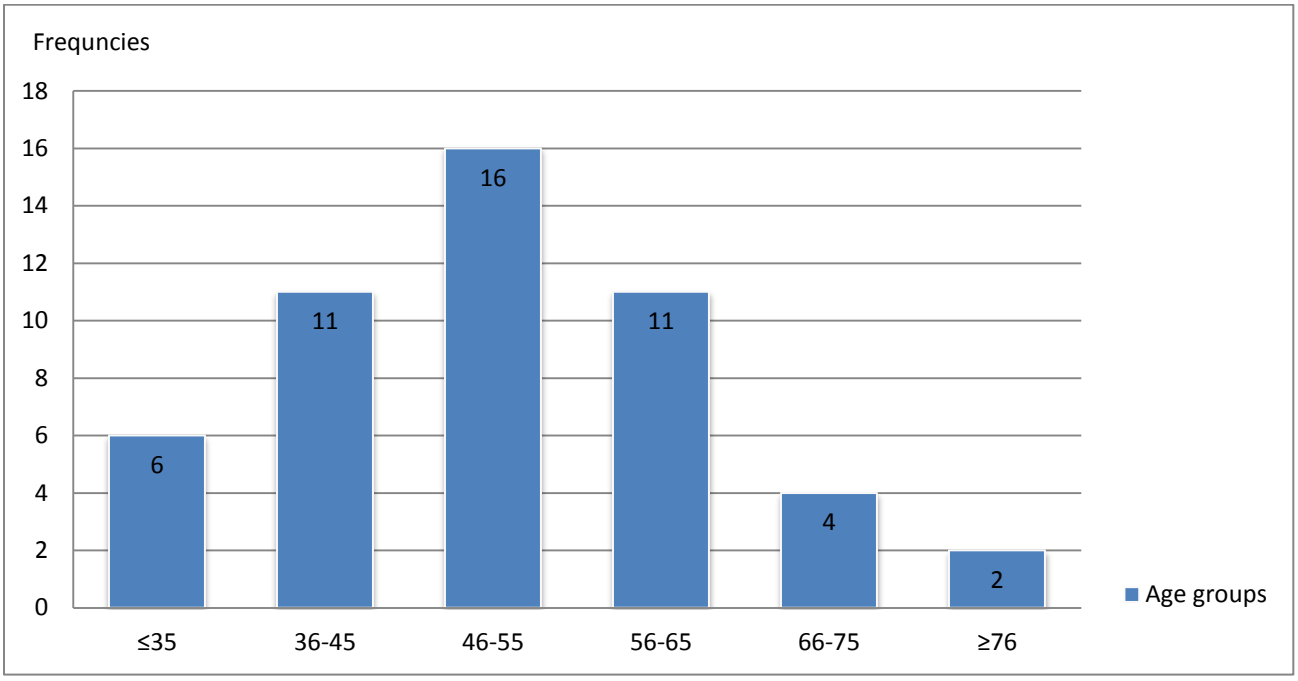


Fig. 4.1 Age group of breast cancer patients

Regarding types of breast cancer (BC) for these patients were mostly invasive ductal 94% (47/50), ductal carcinoma in situ 2% (1/50), mucinous 2% (1/50) and an infiltrating lobular 2% (1/50) (Fig.4.2).

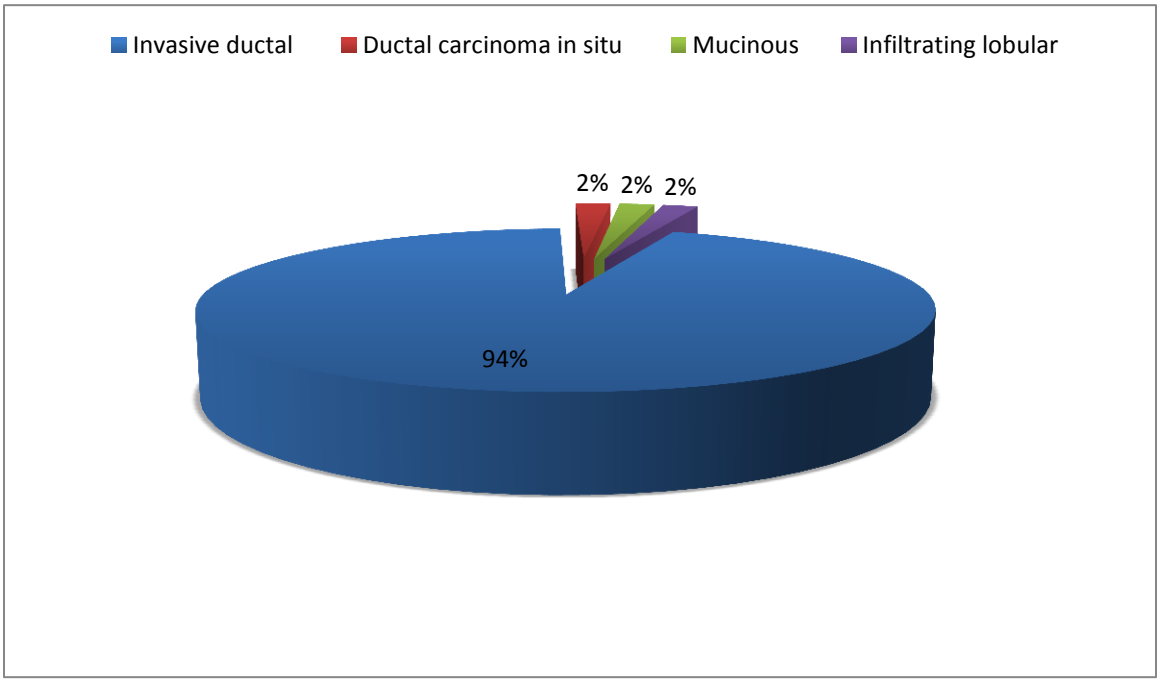


Fig. 4.2 Distribution of study population (n=50) regarding type of breast cancer

The percentage of grade I in all patients is 8% (4/50), grade II 62% (31/50) and grade III 30% (15/50) (Fig. 4.3).

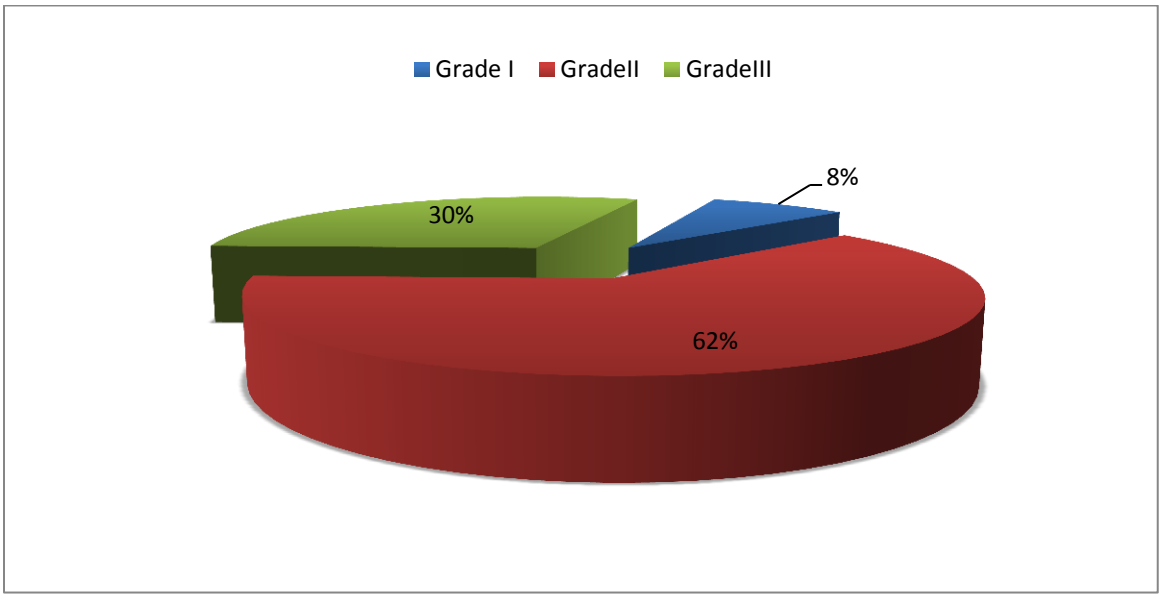


Fig. 4.3 The percentage of grades for the breast cancer patient's

HPV 18 was identified in two samples (4% ; 2/50). HPV 16 was not detected (Fig. 4.4).

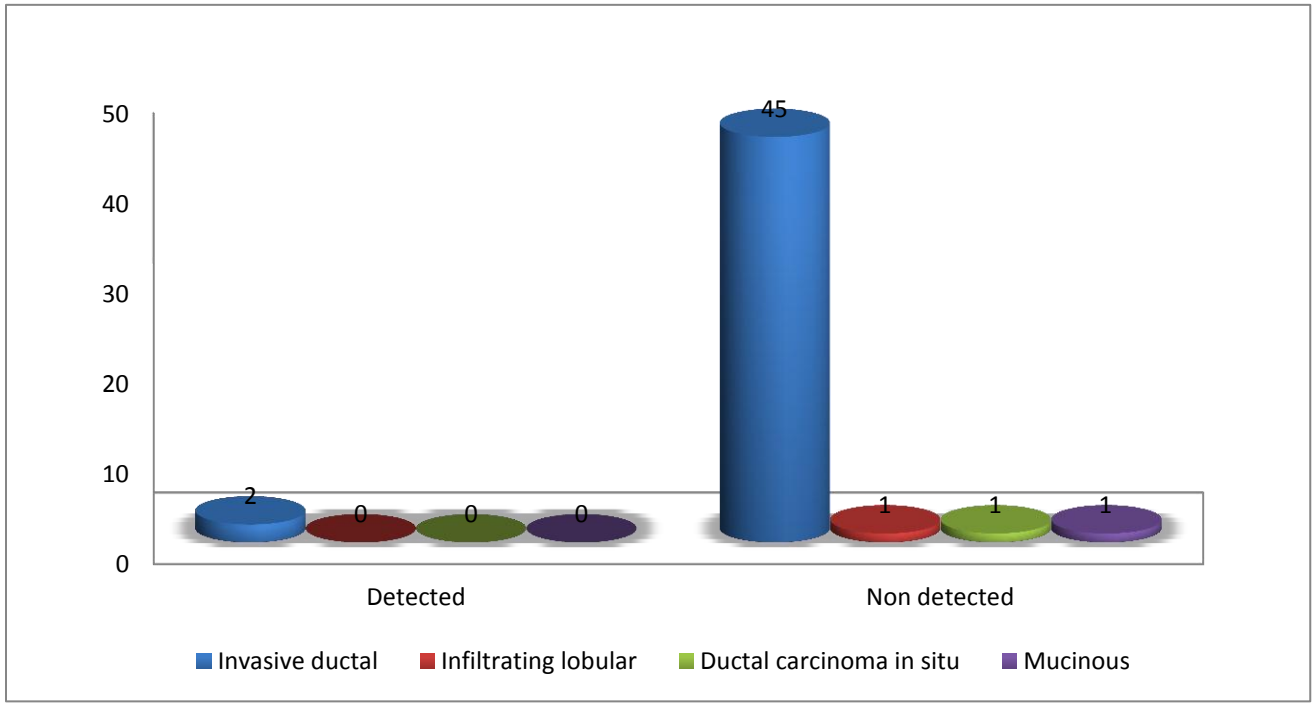


Fig. 4.4 Frequency of HPV 18 among study population in relation to type of breast cancer

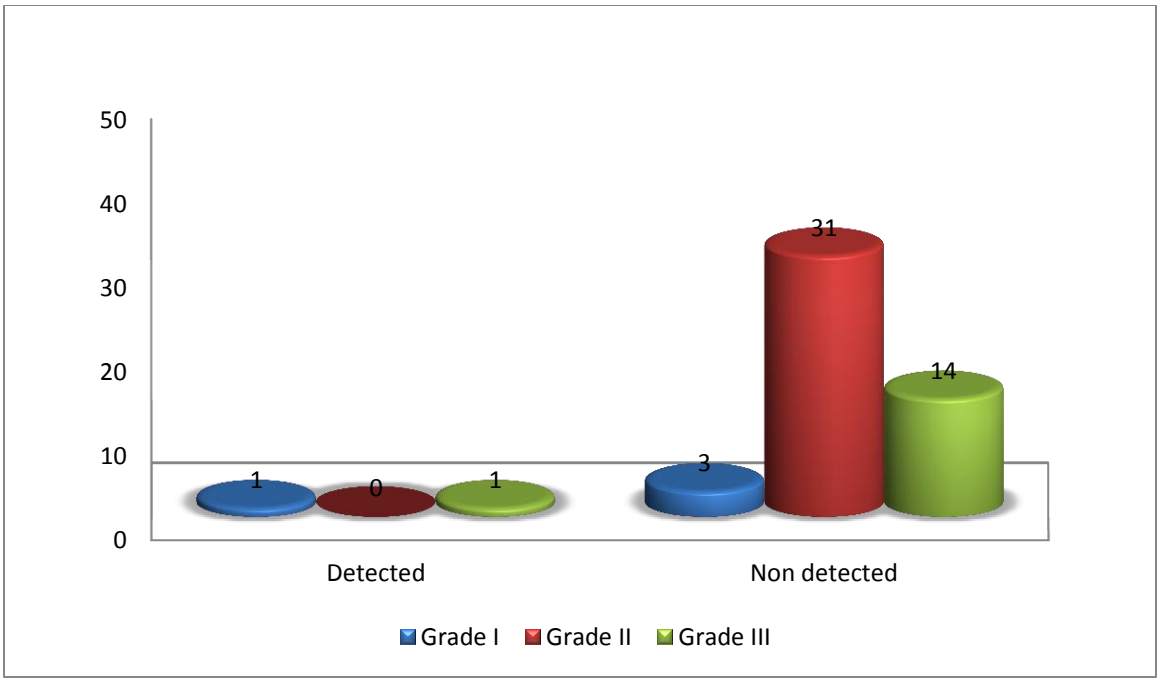


Fig. 4.5 Frequency of HPV 18 among study population in relation to grade of breast cancer

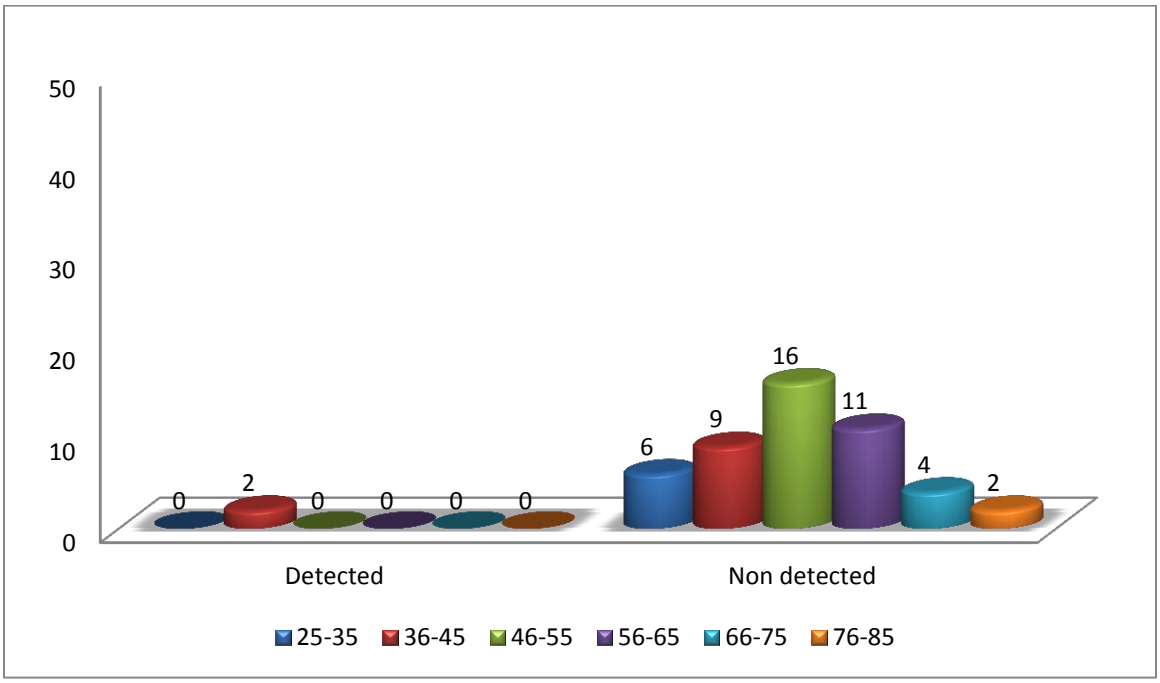


Fig. 4.6 Frequency of HPV 18 among study population in relation to group of age patients

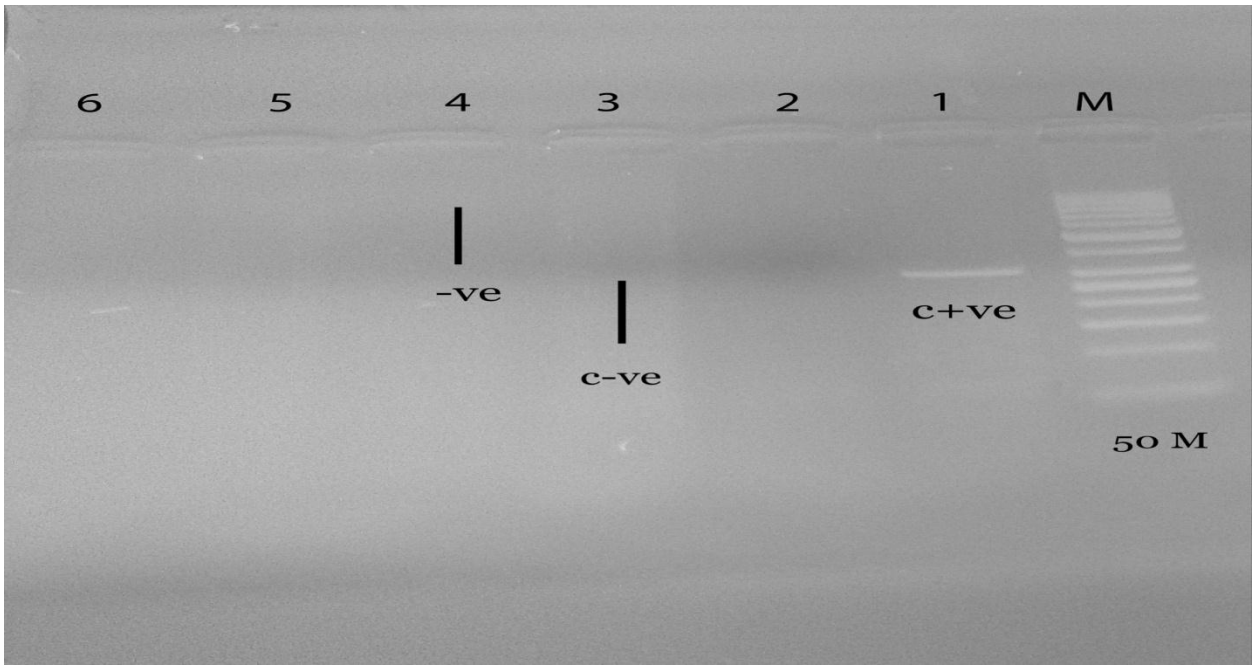


Fig. 4.1 Electrophoresis of representative PCR products of HPV 16 on 2.0% agarose gel. C: Control , M: DNA Marker

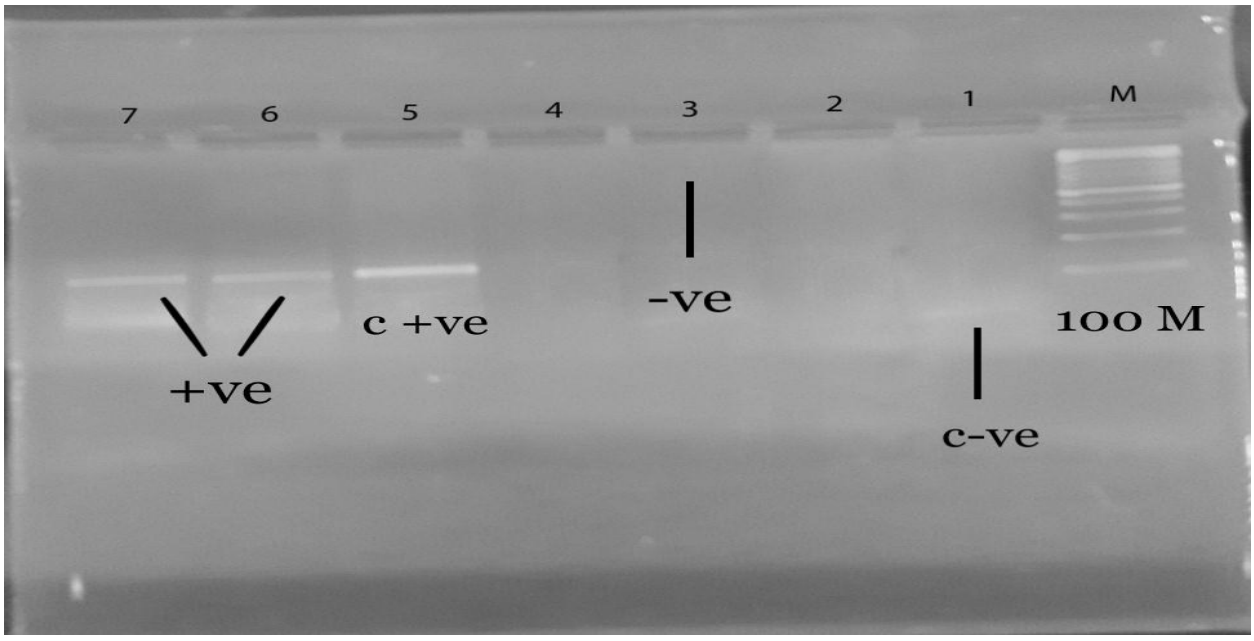


Fig. 4.2 Electrophoresis of representative PCR products of HPV 18 on 2.0% agarose gel. C: Control , M: DNA Marker

CHAPTER FIVE

DISCUSSION

CHAPTER FIVE

5.DISCUSSION

Although the association of human papilloma virus (HPV) with cervical cancer, and head and neck cancers is well established, the involvement of the virus in breast cancer is more controversial.

Fifty formalin embedded tissue blocks previously diagnosed as breast cancer were collected. Only 2/50 (4%) showed diagnostic band for HPV in an invasive ductal carcinoma (IDC), while it was not detected in 48/50 (96%) . The positive band was identified as HPV 18 *E7*, while it was not detected in HPV 16 *E6*. These results are similar to those reported by Li where HPV positive infection was detected in three samples 3/187 (1.6%). Two of them were infected with HPV 18 *E7* while one was infected with HPV 16 *E6* (Li *et al*., 2015).

The present results to be lower than those reported in Brazil where (24.75%) was detected in 25 of breast carcinoma. Out of the 25 positive cases, 14 were HPV-16 positive (56%) and 10 were HPV-18 positive (40%) using primer sets targeting *E6* region (Damin *et al*., 2004).

Also higher results were obtained in a study in Greece which was based on the detection of HPV-DNA from 35 liquid cytology specimens (fine needle aspirates of women with breast cancer) . HPV- DNA was detected in 17.14% of the carcinoma cases and HPV16 DNA was present in 83.3% of them. These results are due to the use of liquid specimens and in situ hybridization technique as confirmatory test (Divani and Giovani, 2012). One of the newest studies published in UK ; which was detected 12 high risk (HR) – HPV genotypes (16, 18, 31, 35, 39, 45, 52, 56, 59,66) from 110 fresh abnormal breast tissue (including cancerous and non-cancerous samples), the results demonstrated that HR-HPV DNA samples were

detected in 46/110 (42%) of the abnormal and normal breast cases . Samples positive for HPV-DNA were screened for viral oncoprotein *E7* expression which was found in an invasive ductal carcinoma. These findings referred to fresh samples which were investigated for HPV DNA by PCR and Sanger method while Western blot and dot blot were used for screening of viral oncoprotein *E7* (Salman *et al*., 2017).

In a study in Australia, 30 (3.5%) low-risk and 20 (2.3%) high-risk HPV types were identified in 855 breast cancers. The high risk types were HPV 18 (48%), HPV 113 (24%), HPV 16 (10%), HPV 52 (10%). HPV type 18 was the most common type identified in breast cancer specimens (55% of 40 breast cancer specimens) followed by HPV 16 (13%) (Lawson *et al*., 2015).

A study in China from fresh frozen samples HPV was found in 4/62 , (6.5%) samples of breast cancer . Three of the four positive samples were HPV 16 positive (75%) , while only was HPV 18 (25%) (Mou *et al.*, 2011).

However in a study in China it was reported that HPV was not associated with breast cancer since it was absent in patients with breast tumors in North-West China (Chang *et al.*, 2012).

From these previous studies HPV 16 was found to be the most frequent genotype causing breast cancer, followed by HPV 18 which suggest that HPV 16 and 18 have an important role in the development of breast cancer. Although HPV 16 was most frequently reported in various studies of breast cancer worldwide, it was only reported in specimens other than breast cancer in Sudan.

The HPV 16 genotype was reported in Sudan in various studies in different frequencies. For example, HPV 16 was the most prevalent type in head and neck cancer which was detected in 3/150 (2%) (Ahmed *et al.*, 2017). HPV 16 was detected in 25/102 (24.5%) suffering from esophageal cancer (Ahmed *et al*., 2017). Moreover, HPV 16 was reported in 10/95 (10.53%) of patients having upper

respiratory and digestive tracts squamous cell carcinoma (Husain *et al*., 2012). Furthermore, HR-HPV subtype 16 was detected in 13/78 (16.7%) of cervical cancer (Eltahir *et al*., 2012).

Despite being reported elsewhere the frequency of HPV 18 was very low in this study in addition to the absence of HPV 16 which was not found in this study. All this might be attributed to the variation in PCR primers selected in various studies as well as molecular techniques. Further research with freshly prepared specimens might yield more significant results.

CHAPTER SIX

CONCLUSION AND RECOMMENDATION

CHAPTER SIX

6.CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

In this study a total of 50 samples from Sudanese Women with breast cancer were testeds and HPV was detected in 2/ 50 (4%), while 48/50 (96%) showed negative results, all were negative for type 16. The positive results were showed at an invasive ductal carcinoma.

6.2 Recommendations

Use more than one technique such as ISH to compare results . Also benign specimens included to detect relation between HPV and breast cancer. Further studies with larger samples size are required to confirm these results.

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APPENDICES

APPENDICES

Appendix I

Lab Instruments

- Autoclave: used for sterilization.
- Incubater: incubation of tissues after adding protinase K.
- Sensitive balance: weight chemicals and reagents.
- Distiller: production of distilled water.
- Refrigerator and deep freezer: preservation samples and primers
- Centrifuge: for centrifugation.
- Pipettes.
- Micowave: dissolve the agarose powder.
- Casting tray: loading the gel.
- Voltage Source.
- Documentation system: provid the UV light.

Appendix II

Chemicals and reagents

- Xylene.
- Ethanol (100%,80%,70%,50%).
- dH_2O.
- Nucleic lysis buffer.
- Protinase K.
- RNase.
- NaCl (saturated)
- Primers (E6&E7)
- Maxime PCR PreMix Kits.*
- Ethidium bromide.
- 10X Tris Boric EDTA buffer (TBE buffer).
- 1X Tris Boric EDTA buffer.
- TE buffer.
- DNA ladder marker

*** Maxime PCR PreMix (*I*- Taq)**

Component in	20µl reaction
i-Taq™ DNA Polymerase(5U/µl)	2.5U
dNTPs	2.5mM each
Reaction buffer(10x)	1x
Gel loading buffer	1x

Appendix III

Preparations of Solutions

(1) Ethanol 500 ml

500 ml ethanol (ice cold or -20℃) 100%

400 ml ethanol + 100 ml dH_2O 80%

350 ml ethanol + 150 ml dH_2O 70%

250 ml ethanol + 250 ml dH_2O 50%

(2) Nucleic Acid Lysis buffer 100 ml dH_2O

Tris Base (MW. 12.14g) 0.12g

NaCl (MW. 58.44g) 2.3g

Na_2EDTA (MW. 74.4g) 0.07g

SDS 0.7g

(3) NaCl (saturated 6M): 100 ml dH_2O

NaCl (MW. 58.44g) 35.1g

(4) 10X Tris Boric EDTA buffer (TBE buffer) 1 liter dH_2O

Tris Base (MW. 12.14g) 48.4g

Boric Acid (MW) 55g

Na_2EDTA (MW. 74.4g) 7.44g

* 1X TBE buffer:

10 ml 10X TBE + 90 ml dH_2O.

(5) TE Buffer

Tris-HCL	10mM
EDTA	1mM

(6) Agarose Gel 2%

One gram from agarose powder was measured and dissolved in 50 ml from 1X TBE buffer then microwaved for 2 min until agarose completely dissolved . Added 2µl from ethidium bromide after cooling. Then the agarose was poured into gel tray with the well comb in place. Placed newly poured gel at room temperature for 20-30 min, until completely solidified.

(7) loading buffer

1X TBE buffer	50 ml

(8) Ethidium Bromide solution

Ten milligrams of ethidium bromide powder were dissolved into 500 µl deionized water, and kept into brown bottle.

(9) Primers (E6&E7 forward and reverse)

The stock solutions were prepared by adding 300µl from dH_2O then were pelleted at 14,000 rpm for 5 min and placed at 4 ℃ over night. The working solutions were prepared by adding 10µl from stock solutions and 90µl from dH_2O then mixed and placed at -20 ℃.

Appendix IV

Protocol of DNA Extraction

Genomic Medicine Biorepository

GMB006

GMB

Extraction and Isolation of DNA from Paraffin-Embedded Tissue

Extraction of core

1. If you intend to isolate DNA from both normal and tumor tissue, one must first prepare an H&E stain from a top slide of the tissue in the block. This slide should be read by an experienced pathologist, and the normal/tumor regions marked accordingly. Once this is done, you can use the slide to identify the corresponding regions in the block. Depending on the aim of the project, it may also be acceptable to use any or all parts of the tissue in the block.
2. Using a 14G needle as your cutting tool, pierce the block in the region of interest, and cut out a 1 to 3 mm core by turning the needle in the block. The depth of the cut should be sufficient to completely pierce the tissue. If you are unable to use the coring method, and can use DNA extracted from any part of the tissue, you can cut 6-8 10 um sections on the microtome and use for DNA extraction. Remember to wipe the stage with alcohol and replace the blade after each block, in order to avoid cross contamination.
3. Carefully transfer the newly cut core or shavings into either a 2 ml polypropylene microcentrifuge tube, or a 15 ml polypropylene centrifuge tube. Label the tube appropriately.
4. Repeat the coring or cutting process as necessary. Place each core or shaving into a separate tube.
5. At a later time, it is recommended that you seal the block with melted paraffin.

Removal of Paraffin

(Note: Xylene is a hazardous chemical. The step 6 must be done in a fume hood, and the resulting waste handled appropriately.)

6. Treat the core via the following steps in 2.0 ml polypropylene microcentrifuge tubes (15 ml polypropylene centrifuge tubes work as well – adjust centrifugation accordingly):
   a. Xylene, 1 ml for 10 minutes – pellet at 14,000 rpm for 10 min – aspirate supt.
   b. Repeat
   c. 100% Ethanol, 1 ml for 10 minutes – pellet at 14,000 rpm for 10 min – aspirate supt.
   d. Repeat
   e. 80% Ethanol, 1 ml for 10 minutes – pellet at 14,000 rpm for 10 min – aspirate supt.
   f. Repeat
   g. 50% Ethanol, 1 ml for 10 minutes – pellet at 14,000 rpm for 10 min – aspirate supt.
   h. Repeat
7. Add 1 ml H_2O and incubate at 4°C overnight.

Digestion of Protein

8. Pellet sample at 14,000 rpm for 10 minutes – aspirate supt.
9. Add 700 ul of Nucleic Acid Lysis buffer (NALB).
10. Add 50 ul of Proteinase K (@ 30 mg/ml).
11. Incubate for 24 hours at 65°C.
12. Add an additional 50 ul of Proteinase K (@ 30 mg/ml).

Protocol GMB006rB: 060205REW
Revised and approved 2010DEC29 by N. Prescott

13. Incubate for an additional 24 hours at 65°C.

Precipitation & Isolation of DNA

14. If the sample requires the elimination of endogenous RNA (recommended), add 10 ul of RNAse A (10mg/ml) and incubate at 37°C for 30 minutes.
15. Add 250 ul of 6 M NaCl (saturated).
16. Let stand at room temp for 10 minutes.
17. Pellet sample at 14,000 rpm for 10 minutes.
18. Carefully transfer supernatant to a clean microcentrifuge tube.
19. Add 1 ml of ice-cold (or –20°C) 100% Ethanol.
20. Carefully mix and place at –20°C for 20 minutes.
21. Pellet sample at 14,000 rpm for 10 minutes.
22. Carefully discard the supernatant.
23. Wash pellet with 1.5 ml of 70% Ethanol.
24. Pellet sample at 14,000 rpm for 10 minutes.
25. Carefully discard the supernatant.
26. Allow pellet to air dry on the benchtop for 10 to 15 minutes.
27. Add 30 to 80 ul of TE Buffer.

Nucleic Acid Lysis Buffer
10 mM Tris Base (1.21 g/L)
400 mM NaCl (32.4 g/L)
2 mM Na_2EDTA (0.75 g/L)
0.7% SDS (7.0 g/L)

Maxime PCR PreMix Kit (*i*- Taq)

Maxime PCR PreMix Series *Research Use Only*

ISO 9001/14001 Certified Company

Maxime PCR PreMix Kit (*i*-Taq)

for 20μl rxn / 50μl rxn

Cat. No. 25025(for 20μl rxn, 96 tubes) **Cat. No. 25026**(for 20μl rxn, 480 tubes)
Cat. No. 25035(for 50μl rxn, 96 tubes)

DESCRIPTION

iNtRON's *Maxime* PCR PreMix Kit has not only various kinds of PreMix Kit according to experience purpose, but also a 2X Master mix solution.
Maxime PCR PreMix Kit (*i*-Taq) is the product what is mixed every component: *i*-Taq™ DNA Polymerase, dNTP mixture, reaction buffer, and so on- in one tube for 1 rxn PCR. This is the product that can get the best result with the most convenience system. The first reason is that it has every components for PCR, so we can do PCR just add a template DNA, primer set, and D.W.. The second reason is that it has Gel loading buffer to do electrophoresis, so we can do gel loading without any treatment. In addition, each batches are checked by a thorough Q.C., so its reappearance is high. It is suitable for various sample's experience by fast and simple using method.

STORAGE

Store at -20°C; under this condition, it is stable for at least a year.

CHARACTERISTICS

- High efficiency of the amplification
- Ready to use: only template and primers are needed
- Stable for over 1 year at -20℃
- Time-saving and cost-effective

CONTENTS

- *Maxime* PCR PreMix (*i*-Taq, for 20μl rxn) 96 (480) tubes
- *Maxime* PCR PreMix (*i*-Taq, for 50μl rxn) 96 tubes

Component in	20 μl reaction	50 μl reaction
i-Taq™ DNA Polymerase(5U/μl)	2.5U	5U
dNTPs	2.5mM each	2.5mM each
Reaction Buffer(10x)	1x	1x
Gel Loading buffer	1x	1x

Note : The PCR process is covered by patents issued and applicable in certain countries. iNtRON Biotechnology does not encourage or support the unauthorized or Unlicensed use of the PCR process. Use of this product is recommended for persons That either have a license to perform PCR or are not required to obtain a license.

PROTOCOL

1. Add template DNA and primers into *Maxime* PCR PreMix tubes (*i*-Taq).
   Note 1 : Recommended volume of template and primer : 3μl~9μl
   Appropriate amounts of DNA template samples
   - cDNA : 0.5-10% of first RT reaction volume
   - Plasmid DNA : 10pg-100ng
   - Genomic DNA : 0.1-1ug for single copy

   Note 2 : Appropriate amounts of primers
   - Primer : 5-20pmol/μl each (sense and anti-sense)
2. Add distilled water into the tubes to a total volume of 20μl or 50μl . Do not calculate the dried components

Example **Total 20μl or 50μl reaction volume**

PCR reaction mixture	Add	Add
Template DNA	1 ~ 2μl	2 ~ 4μl
Primer (F : 10pmol/μl)	1μl	2 ~ 2.5μl
Primer (R : 10pmol/μl)	1μl	2 ~ 2.5μl
Distilled Water	16 ~ 17μl	44 ~ 41μl
Total reaction volume	**20 μl**	**50 μl**

Note : This example serves as a guideline for PCR amplification. Optimal reaction conditions such as amount of template DNA and amount of primer, may vary and must be individually determined.

3. Dissolve the blue pellet by pipetting.
   Note : If the mixture lets stand at RT for 1-2min after adding water, the pellet is easily dissolved.
4. (Option) Add mineral oil.
   Note : This step is unnecessary when using a thermal cycler that employs a top heating method(general methods).
5. Perform PCR of samples.
6. Load samples on agarose gel without adding a loading-dye buffer and perform electrophoresis.

SUGGESTED CYCLING PARAMETERS

PCR cycle		Temp.	PCR product size		
			100-500bp	500-1000bp	1Kb-5Kb
Initial denaturation		94℃	2min	2min	2min
30-40 Cycles	Denaturation	94℃	20sec	20sec	20sec
	Annealing	50-65℃	10sec	10sec	20sec
	Extension	65-72℃	20-30sec	40-50sec	1min/Kb
Final extension		72 ℃	Optional. Normally, 2-5min		

EXPERIMENTAL INFORMATION

- ***Comparison with different company kit***

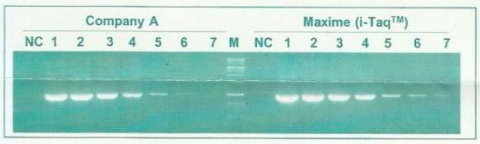

Fig.1. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 1 Kb DNA fragment .
After diluting the λDNA as indicates, the PCR reaction was performed with *Maxime* PCR PreMix (*i*-Taq) and company's A product.
Lane M, SiZer-1000 DNA Marker; **lane 1,** undiluted λDNA; **lane 2,** 200 ng λDNA; **lane 3,** 40 ng λDNA; **lane 4,** 8 ng λDNA; **lane 5,** 1.6 ng λDNA; ; **lane 6,** 320 pg λDNA; **lane 7,** 64 pg λDNA; **lane NC**, Negative control

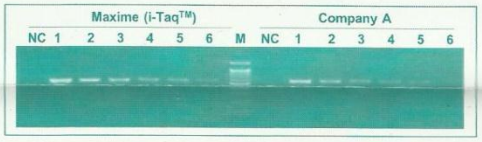

Fig.2. Comparison of *Maxime* PCR PreMix (*i*-Taq) and Company A's PreMix system by amplifying 570 bp DNA fragment (GAPDH).
Total RNA was purified from SNU-1 using easy-BLUE™ Total RNA Extraction Kit (Cat. No. 17061). And then, the first strand of cDNA was synthesized using Power cDNA Synthesis Kit (Cat. No. 25011). After diluting the cDNA mixture as indicates, the RT-PCR reaction was performed.
lane M, SiZer-100 DNA Marker; **lane 1,** undiluted cDNA; **lane 2,** 1/2 diluted cDNA; **lane 3,** 1/4 diluted cDNA; **lane 4,** 1/8 diluted cDNA; **lane 5,** 1/16 diluted cDNA; **lane 6,** 1/32 diluted cDNA; **lane NC**, Negative control

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